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**14. ABSTRACT**
The primary objective of this proposal was to identify perineural drug combinations that enhance pain relief by producing long-duration, sensory-specific nerve block with minimal toxicity. We assessed the efficacy of the adjuvants clonidine (C), buprenorphine (B), dexamethasone (D), and midazolam (M), alone and in combination with local anesthetics (LAs), in the block of peripheral nerve in vitro (Aim 1) and in vivo (Aim 2). Results of the first set of in vitro experiments indicated that M blocked C-fibers with greater potency and efficacy than A-fibers, consistent with a sensory-specific mode of action. C blocked A- and C-fibers, but only at concentrations greater than those associated with systemic side effects in vivo. D and B blocked neither C- nor A-fibers at clinically relevant concentrations. The combination of B, C and D had no influence on either LA- or M-induced nerve block. Further analysis of M effects indicated that peripheral nerve block and toxicity were due to a benzodiazepine receptor-independent increase in intracellular Ca2+. That increased axonal K+ conductance may be a viable way to increase LA potency was confirmed with several different classes of K+ channel opener in the isolated nerve preparation. Nevertheless, because the other adjuvants prolong LAinduced block in vivo, the failure to detect an influence of this drug combination on isolated nerves in vitro suggests that the drug interaction involves mechanisms extrinsic to peripheral nerve axons.

**15. SUBJECT TERMS**
Nerve block; ropivacaine; dexamethasone; clonidine; buprenorphine; midazolam; compound action potential

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**Table of Contents**

**INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research**.**

Trauma-related acute pain is common among injured soldiers. Untreated acute pain is a reliable predictor of chronic pain. Peripheral nerve blocks provide exceptional pain relief for traumatic injuries, while (i) avoiding various central nervous system side effects (such as sedation and respiratory depression), (ii) facilitating evacuation of injured troops from the field of combat, and (iii) providing therapies that, when repeated, help with recovery. However, long duration (>12-24 hr) blocks can only be achieved, at present, with nerve block catheters that are both labor-intensive and technically difficult to insert. Furthermore, local anesthetics block both sensory and motor axons, thereby limiting the ability of patients (after surgical correction) to fully engage in physical therapy while increasing the risk of falls.

As detailed in previous Annual Reports, we completed a series of *in vitro* experiments using the isolated rat sciatic nerve to examine direct effects of the adjuvants clonidine (C), buprenorphine (B), dexamethasone (D) and midazolam (M) applied alone and in combinations on propagated action potentials. The local anesthetic bupivacaine was highly efficacious, but we found no direct effect of D or B alone on propagated A- or C-fiber action potentials in the sciatic nerve at concentrations used clinically. Clonidine exerted a modest effect and M produced an apparent selective, limited block of C-fiber propagation. When studied in combinations with bupivacaine, neither C, B, D or M influenced either the potency or duration of local anesthetic-induced block of A- or C-fiber action potentials. Interestingly, M appeared to exacerbate the neurotoxic effects of local anesthetics on sensory neurons isolated from dorsal root ganglia.

These results have been published (*1*), suggesting [1] that the clinical reports of adjuvants prolongation of the duration of local anesthetic-induced block of peripheral nerves arises from either indirect mechanisms or mechanisms not directly associated with the nerve itself (e.g., regulation of immune cells, blood flow, sympathetic innervation, etc) and [2] further experiments. Accordingly, we undertook investigation into the mechanism(s) responsible for M-induced nerve block and neurotoxicity, hoping to identify a means to produce a nerve block in the absence of toxicity. We also hypothesized that potassium channel openers might directly potentiate local anesthetic induced peripheral nerve block, again hoping to use this strategy as a means to produce a selective nerve block as well.

The results from the M-induced neurotoxicity study has been prepared for publication and will be submitted shortly. Results from the potassium channel opener study failed to support the hypothesis that the duration of peripheral nerve block would be significantly extended. A manuscript describing the results of this final study is in preparation.

**BODY**

For convenience, *the most recent Statement of Work narrative is* ***copied and pasted as boldfaced italicized text***. After the italicized text, normal-font text follows with a progress report of each section / subsection. The Statement of Work referenced is dated November 7, 2011.

***Task 1. Isolated in vitro sciatic nerve preparations for the measurement of compound action potentials (CAP)***

Experiments **1a** - **c** were completed during the first year of this award and were described in detail in our first annual report. These results are fully described in a manuscript (*1*).

***1d. Monotherapy on compound action potentials (CAPs) in presence of retigabine and its associated control experiments and***

***1e. Combination therapy on CAPs using retigabine with lidocaine (L)***

As indicated in the Introduction, we hypothesized that potassium channel openers might directly potentiate local anesthetic induced peripheral nerve block and thus initiated experiments (**1d**) with retigabine, a KNCQ/M-type $K^+$ channel opener currently used in Europe as an anti-seizure medication. The first experiment utilized the isolated rat sciatic nerve to evaluate whether retigabine affected either or both the A- and C-fiber propagated waves of action potentials. At the highest concentration of retigabine tested (300 μM), there was an increase in conduction velocity of both A- and C-waves, consistent with membrane hyperpolarization associated with the activation of $K^+$ channels; there was no evidence of compound action potential block. Interestingly, retigabine given in combination with lidocaine (**1e**) significantly increased the time of recovery from block without affecting the potency of the lidocaine-induced block. We then tested other concentrations of retigabine in combination with lidocaine, and combined concentrations that produced ~50% block of the compound action potential resulted in compound action potential blockage for more than 8 hrs – that is, there was no washout and no recovery, which usually takes ~30 minutes. When we examined whether this combination of concentrations was toxic, we noted a significant increase in toxicity and thus undertook further dose-finding experiments to determine whether there existed a non-toxic retigabine-lidocaine combination that produced a long lidocaine-induced block of the compound action potential. We found that it was possible to roughly triple the duration of lidocaine-induced compound action potential nerve block with a concentration of retigabine (100 nM) that was well below the concentration that significantly increased toxicity.

***1f. Mono- and combination therapies using benzodiazepine substrates***

In these experiments, we followed-up on the observation that M produced a block of the compound action potential with greater potency for C-wave than that for block of the A-wave, addressing concerns about neurotoxicity (*2*). Midazolam is a benzodiazepine receptor agonist with equal potency at central (*3, 4*) and peripheral (*5*) receptors and we hypothesized that M-produced toxicity and nerve block were effected at different receptors, hoping to maximize the therapeutic utility of M in blocking the C-wave while minimizing or eliminating its neurotoxicity. To test this hypothesis, we tested the effects of M in the presence of benzodiazepine receptor antagonists with central [flumazenil, (*3*)] or the peripheral [PK11195 (*5*)] sites of action. Neither antagonist had any effect on neuronal toxicity or compound action potential propagation and neither antagonist affected the nerve blocking or neurotoxic actions of M, suggesting that both nerve blocking and neurotoxic actions of M are unrelated to interaction with benzodiazepine receptors (i.e., are "off-target" action). In experiments to interrogate the mechanism(s) of M effects, we focused on intracellular $Ca^{2+}$ and whether M-produced neurotoxicity arose from M's ability to increase intracellular $Ca^{2+}$. Midazolam did produce a concentration-dependent increase in intracellular $Ca^{2+}$ that was unaffected by either of the benzodiazepine receptor antagonists, thus confirming its off-target effect on intracellular $Ca^{2+}$. This suggested testing a $Ca^{2+}$ chelator, BAPTA, which not unexpectedly attenuated the M-induced block of the compound action potential. A manuscript containing the results of this series of experiments is currently in preparation and should be submitted for publication by the end of the calendar year.

***Task 2. In vivo rat sciatic nerve procedures and behavioral experiments***

***Aim 2. Analgesia and immobilization using drugs tested in experiments 1d-1e***

Following-up on the results obtained with retigabine described above, we tested retigabine given alone or in combination with ropivacaine as a perineural injection in rats. Thermal (heat) and mechanical stimuli were used for nociceptive testing, and the rotorod test was used to assess motor effects. The results of these in vivo experiments failed to show that retigabine in combination with ropivacaine significantly increased the duration of antinociception/nerve block produced by ropivacaine alone (aim 2a). The magnitude of effect was significantly less than observed in vitro. We noted no apparent toxicity produced by the combination (aim 2d), but also no worsening of motor block by addition of retigabine to the ropivacaine (aim 2b). Finally, we tested whether a drug used to activate $Ca^{2+}$ dependent $K^+$ channels (NS1619) in combination with ropivacaine would improve the duration or magnitude of local anesthetic block. Preliminary results were negative.

See report by Dr. Williams for progress on Task 3: “Scholarly and other required regulatory, writing, and publication tasks.”

**KEY RESEARCH ACCOMPLISHMENTS:** *Bulleted list of key research accomplishments emanating from this research.*

- Publication in the peer-reviewed literature demonstrating that adjuvants currently in clinical use to prolong the duration of local anesthetic-induced regional anesthesia do so by mechanisms extrinsic to the peripheral nerve itself (*1*).
- Finding that retigabine, a $K^+$ channel opener, significantly increases the duration of lidocaine-induced block of action potential propagation in the isolated peripheral rat sciatic nerve at concentrations that produce minimal neurotoxicity.
- Determination that neither the nerve block nor neurotoxicity associated with M is due to actions at peripheral or central benzodiazepine receptors. Rather, we determined that these effects of M are due to an increase in intracellular $Ca^{2+}$.
- Finding that retigabine and ropivacaine given in combination produces a significantly longer duration of nerve block analgesia than 1] produced by ropivacaine alone and 2] without significant effects on motor function.

**REPORTABLE OUTCOMES:** *Provide a list of reportable outcomes that have resulted from this research to include: manuscripts, abstracts, presentations; patents and licenses applied for and/or issued; degrees obtained that are supported by this award; development of cell lines, tissue or serum repositories; informatics such as databases and animal models, etc.; funding applied for based on work supported by this award; employment or research opportunities applied for and/or received based on experience/training supported by this award*

**Publication:** Yilmaz-Rastoder E, Gold MS, Hough KA, Gebhart GF, Williams BA: Effect of adjuvant drugs on the action of local anesthetics in isolated rat sciatic nerves. *Reg Anesth Pain Med* **37**: 403-409, 2012 (with editorial).

Two additional manuscripts are in preparation for publication in the peer reviewed literature.

*There are no other reportable outcomes, as identified in the instructions.*

**CONCLUSION:** *Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what" section which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.*

In evaluating whether nerve block analgesia is extended by using perineural adjuvants clonidine-buprenorphine-dexamethasone, as suggested in the clinical literature, we found that this three-drug combination does not influence the duration of the compound action potentials of isolated peripheral nerve *in vitro*, with or without the co-administration of local anesthetics or midazolam (*1*). We did, however, find that these adjuvants are able to prolong the actions of local anesthetics via a mechanism extrinsic to the peripheral nerve itself, such as resident and recruited immune cells, the local vasculature, and the sympathetic innervation of the peripheral nerve and vasculature.

Midazolam was found to produce a nerve block which, however, is not effected at either peripheral or central benzodiazepine receptors. Instead, the block is due to an “off target” effect associated with an increase in intracellular $Ca^{2+}$ via release from internal stores.

Retigabine effectively increased the duration of local anesthetic induced block of peripheral nerves via an activation of $K^{+}$ channels in the peripheral nerve.

***“So What?”:*** Our findings have three implications.

1. The results of the published experiments suggest that extrinsic factors may be appropriate targets for development of more effective and selective adjuvants which may prolong the duration modality of regional anesthesia with minimal side effects.
2. The mechanisms of action uncovered and underlying M-induced modality block of peripheral nerve suggests additional strategies to produce modality specific block by mechanisms intrinsic to the peripheral nerve.
3. The results of experiments with retigabine suggest that a compound already approved for use in humans (in Europe) may be useful to achieve an important goal of this project, namely to generate a long duration, modality selective block of the peripheral nerve.

**REFERENCES:** List all references pertinent to the report using a standard journal format (i.e., format used in *Science, Military Medicine*, etc.).


1. E. Yilmaz-Rastoder, M. S. Gold, K. A. Hough, G. F. Gebhart, B. A. Williams, *Reg Anesth Pain Med* **37**, 403 (Jul-Aug, 2012).
2. B. A. Williams *et al.*, *Reg Anesth Pain Med* **36**, 225 (May-Jun, 2011).
3. C. S. Goodchild, J. M. Serrao, *Br J Anaesth* **59**, 1563 (Dec, 1987).
4. T. Kohno *et al.*, *Anesthesiology* **104**, 338 (Feb, 2006).
5. Z. Zhao, L. Hertz, W. E. Code, *Can J Physiol Pharmacol* **74**, 273 (Mar, 1996).

ORIGINAL ARTICLE

# Effect of Adjuvant Drugs on the Action of Local Anesthetics in Isolated Rat Sciatic Nerves

*Eser Yilmaz-Rastoder, MS,*† Michael S. Gold, PhD,*† Karen A. Hough, AS, CVT, RLAT,*† G.F. Gebhart, PhD,*† and Brian A. Williams, MD, MBA*†‡*

**Background and Objectives:** There is increasing clinical use of adjuvant drugs to prolong the duration of local anesthetic–induced block of peripheral nerves. However, the mechanistic understanding regarding drug interactions between these compounds in the periphery is quite limited. Accordingly, we undertook this study to determine whether selected adjuvant drugs are efficacious in blocking action potential propagation in peripheral nerves at concentrations used clinically and whether these drugs influence peripheral nerve block produced by local anesthetics.

**Methods:** Isolated rat sciatic nerves were used to assess (1) the efficacy of buprenorphine, clonidine, dexamethasone, or midazolam, alone and in combination, on action potential propagation; and (2) their influence on the blocking actions of local anesthetics ropivacaine and lidocaine. Compound action potentials (CAPs) from A- and C-fibers were studied before and after drug application.

**Results:** At estimated clinical concentrations, neither buprenorphine nor dexamethasone affected either A- or C-waves of the CAP. Clonidine produced a small but significant attenuation of the C-wave amplitude. Midazolam attenuated both A- and C-wave amplitudes, but with greater potency on the C-wave. The combination of clonidine, buprenorphine, and dexamethasone had no influence on the potency or duration of local anesthetic– or midazolam-induced block of A- and C-waves of the CAP.

**Conclusions:** These results suggest that the reported clinical efficacy of clonidine, buprenorphine, and dexamethasone influences the actions of local anesthetics via indirect mechanisms. Further identification of these indirect mechanisms may enable the development of novel approaches to achieve longer-duration, modality-specific peripheral nerve block.

(*Reg Anesth Pain Med* 2012;37: 403–409)

While there are a wide range of procedures for which short-acting local anesthesia is ideal, it is becoming increasingly clear that long-duration blocks are useful, particularly in the perioperative setting.[1,2] One of several strategies that have been used to increase the duration of local anesthetic block of peripheral nerves is based on the use of adjuvant drugs that, when used in combination with local anesthetics, can prolong the duration of action. Epinephrine has been used most extensively in this capacity.[3] Given the relatively limited efficacy of epinephrine, anesthesiologists have turned to additional compounds, including clonidine, buprenorphine, dexamethasone, and midazolam as single adjuvant drugs or in combinations[1,4–6] despite the fact that the mechanisms underlying the local anesthetic–prolonging actions of these compounds remain to be determined. Furthermore, the efficacy of these adjuvant drugs used in combination with local anesthetics has encouraged anesthesiologists to use them in the absence of local anesthetics,[1,4] in the hope that an optimal combination of adjuvant drugs might have modality selectivity (ie, sensory instead of motor fiber block).

The purpose of this study was 2-fold. First, to determine whether adjuvant drugs now commonly used with local anesthetics in peripheral nerve blocks act directly on the peripheral nerve to block propagation of action potentials; and second, to determine whether there is a direct effect of these adjuvant drugs on the actions of local anesthetics in the block of propagated action potential in the isolated nerve. We hypothesized that (i) adjuvant drugs manifest anesthetic effects on their own at clinical concentrations and (ii) these anesthetic effects are additive when drugs are combined. To test these hypotheses, we recorded compound action potentials (CAPs) from A-fibers (A-CAPs) and C-fibers (C-CAPs) in isolated rat sciatic nerves before and after application of adjuvant drugs alone or in combination with the local anesthetic ropivacaine or lidocaine.

## METHODS

### Animals

Adult male Sprague-Dawley rats (275–350 g; Harlan Sprague Dawley, Indianapolis, Indiana) were used for the experiments. Rats were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited animal care facility with unrestricted food and water on a 12:12-hour light-dark cycle until use. All procedures involving animals were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Pittsburgh, Pennsylvania). Animal care and handling were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

### Drugs

We purchased all except 1 drug in preservative-free injectable solutions in the following concentrations: clonidine HCl 0.1 mg/mL (Duraclon; Xanodyne Pharmaceuticals, Inc, Newport, Kentucky), buprenorphine HCl 0.3 mg/mL (Ben Venue Laboratories, Bedford, Ohio), dexamethasone sodium phosphate 10 mg/mL (preservative-free, with no benzyl alcohol; APP Pharmaceuticals, Schaumburg, Illinois), midazolam HCl (Ben Venue Laboratories) and ropivacaine HCl (Naropin; APP Pharmaceuticals) 5 mg/mL. Epinephrine and lidocaine HCl were purchased in powder form (Sigma-Aldrich, St Louis, Missouri). The

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concentrations of buprenorphine, dexamethasone, epinephrine, midazolam, and ropivacaine used in the present study were based on estimation of concentrations that would be used clinically[6]: 1 μg/mL (3.76 μM) for clonidine, 3 μg/mL (5.95 μM) for buprenorphine, 66.7 μg/mL (130 μM) for dexamethasone, 16.7 μg/mL (51.3 μM) for midazolam, and 2.5 mg/mL (9.13 mM) for ropivacaine. Of note, dexamethasone is often used in a preparation made with 1% benzyl alcohol, and it is possible that influence of this adjuvant on the actions of local anesthetics is due to the alcohol rather than dexamethasone, per se. However, the preservative-free form used in the present study is also used clinically, and there is evidence that this preparation can prolong the actions of local anesthetics.[5,7] We therefore focused on the potential actions of dexamethasone rather than its preservative. The concentration of clonidine used is based on assumptions about infusion rates when the drug was used in clinical studies in combination with local anesthetics,[8,9] as well as clinical observations regarding the sedative effects of clonidine. We therefore attempted to bracket this range in the experiments performed. The concentrations of lidocaine were based on those necessary to block voltage-gated $Na^+$ channels in isolated tissue preparations,[10] which are concentrations far below those used clinically. Adjuvant drugs were tested at concentrations starting 2 to 3 orders of magnitude below the clinical values to at least 1 order of magnitude above the clinically relevant concentration. In the case of ropivacaine, we did not need to exceed the clinical dose because we were able to reach a maximum level of CAP attenuation at lower concentrations.

## Recording CAPs From Isolated Sciatic Nerves

Rats were anesthetized with an intraperitoneal injection of either pentobarbital (60 mg/kg) or 1 mL/kg of a mixture of ketamine (55 mg/mL)/xylazine (5.5 mg/mL)/acepromazine (1.1 mg/mL). Sciatic nerves (~30 mm) were quickly dissected and immediately transferred to a container containing ice-cold Locke solution of the following composition (in mM): 136 NaCl, 5.6 KCl, 14.3 $NaHCO_3$, 1.2 $NaH_2PO_4$, 2.2 $CaCl_2$, 1.2 $MgCl_2$, 11 dextrose, equilibrated continuously with 95% $O_2$, 5% $CO_2$, pH 7.2 to 7.4. Nerves were trimmed of excess connective tissue and kept in ice-cold oxygenated Locke solution for at least 1 hour before use. One end of each nerve was laid over 2 platinum stimulating electrodes in the recording chamber. The central portion of the nerve, separated from the stimulating electrode by a grease gap, was superfused continuously (2–5 mL/min) with oxygenated Locke solution at room temperature with and without drugs delivered via a gravity-driven perfusion system. Compound action potentials were recorded from the other end of the nerve with a glass suction electrode connected to the input stage of a differential preamplifier (0.1–10 kHz; WPI model DAM-80, Sarasota, Florida). Compound action potentials were evoked with supramaximal electrical pulses 0.2 to 0.5 milliseconds in duration, 0.05 Hz, filtered at 2 kHz, and sampled at 20 kHz. Voltage data were digitized via a CED 1401 Micro A/D converter and analyzed using CED Spike 2 version 5 for MS Windows (CED, Cambridge, England). Waveform data were rectified, averaged for 6 consecutive CAPs, and integrated to quantify A- and C-fiber components as area under the curve (AUC). The A-fiber deflection of the CAP (A-wave) was easily distinguished from that associated with the C-fiber deflection (C-wave) because of the time delay between the arrival of the 2 waves at the recording electrode. Specifically, we analyzed CAPs of A-fibers conducting between 15 and 80 m/s and of C-fibers conducting slower than 1.5 m/s. Another wave with a conduction velocity consistent with A-delta fibers was present in many of the nerves studied. However, this wave was not included in subsequent analyses, both because of the variability in its amplitude and because the conduction velocity slowing caused by local anesthetics pushed this wave into the more slowing conducting C-wave, precluding a clear resolution of the impact of adjuvant drugs and local anesthetics. It should be noted that an A-delta wave slowed by LA to the point that it was indistinguishable from the C-wave might have influenced our estimates of the potency of LA-induced block of the C-wave.

## Statistics

The primary end points of this study were 3-fold: (1) to determine the potency and efficacy of drug-induced suppression of the CAP in the rat sciatic nerve, (2) to determine the time course of recovery of drug-induced suppression of the CAP in the rat sciatic nerve, and (3) to determine the influence of adjuvant drugs on the potency and time course of recovery of local anesthetic–induced suppression of the CAP. To minimize the number of animals used in this study, both sciatic nerves were harvested from each rat and randomly assigned to different treatment groups. Each individual nerve was used for a single experiment, unless otherwise stated. Because there seemed to be no consistent differences between nerves studied from each animal relative to the total population of nerves studied, each nerve was treated as an independent observation. Thus, "n" represents the number of nerves rather than the number of animals. Nevertheless, to minimize the potential impact of a within-animal effect, nerves from at least 4 rats were used in each experimental group. To determine sample sizes, power analyses were performed with a 2-way repeated-measures analysis of variance (ANOVA) and a Student *t* test to determine the number of nerves needed for detecting a significant suppression of the CAP relative to control nerve followed over time and the number of nerves needed to detect a significant influence of adjuvant drugs on the potency and time course of recovery of LA-induced CAP block. For the single-adjuvant concentration-response studies, we determined that a sample size of 6 was sufficient to enable us to detect a 25% suppression of the CAP with a power of 0.8 and $\alpha$ at 0.05. Similarly, for the LA studies with and without adjuvant drugs, a sample size of 4 was sufficient to enable us to detect a change in potency of recovery greater than 25% with the same values for power and $\alpha$. In several cases, additional nerves were used to increase our confidence in the negative results we obtained. Area under the curve data from individual nerves were averaged for each group. Potency and efficacy of drug treatments were determined from concentration-response data fitted with a modified Hill equation of the form: $(1 - CAP_{drug}/CAP_{ctrl}) = ([drug]^{nH}(E_{max})/[drug]^{nH} + EC_{50}{}^{nH})$, where $EC_{50}$ is the concentration of drug needed to block 50% of the CAP (a measure of potency), $E_{max}$ is the maximal fractional block of the CAP (a measure of efficacy), and $n_H$ is the Hill coefficient. A mixed-design 2-way ANOVA (time × treatment) was used to assess the impact of increasing concentrations of drug relative to control (choline)-treated nerves. One-way ANOVA was used to assess differences in potency and efficacy between drug treatments. The Student *t* test was used to compare the impact of adjuvant drug combinations with local anesthetic or the impact midazolam had on the fractional block and/or the kinetics of the block (onset or recovery) relative to local anesthetic or to midazolam alone. All pooled data are expressed as a fraction of inhibition, which was determined for each nerve by the following equation: fractional inhibition = $1 - (AUC_{Drug}/AUC_{Baseline})$, where $AUC_{Drug}$ was the area under the curve of the rectified CAP in the presence

of drug and $AUC_{Baseline}$ was the area under the curve of the rectified CAP before the application of test agents.

## RESULTS

### Concentration-Response Curves of Individual Drugs

A total of 106 nerves were used in our experiments. Consistent with the results of previous studies, we observed a concentration-dependent block of both A- and C-waves of the sciatic nerve CAP by ropivacaine and lidocaine (Fig. 1). $EC_{50}$ values for ropivacaine were 0.28 ± 0.04 and 0.18 ± 0.09 mM, for the A- and C-waves, respectively. Values for lidocaine were 0.28 ± 0.05 and 0.31 ± 0.7 mM for the A- and C-waves, respectively. Both drugs were fully efficacious, completely blocking both A- and C-waves.

Of the adjuvant drugs tested, midazolam had the greatest efficacy in attenuating A- and C-waves of the CAP (Fig. 1). The effect of midazolam was concentration-dependent over the concentration range tested. Neither the A-wave nor the C-wave was fully blocked at the highest concentration tested (ie, 10 mM). Interestingly, in contrast to the A-wave, midazolam block of the C-wave seemed to be biphasic, with a high-affinity component apparently saturating at ~35% block of the C-wave, and a lower-affinity component that seemed likely to enable complete block of the C-wave at greater concentrations of midazolam. Both components were significantly ($P = 0.03$) greater than the reduction of the C-wave observed in control nerves.

Previous data support the suggestion that clonidine has local anesthetic properties at high concentrations with an $EC_{50}$ of ~2 and 0.45 mM for block of A- and C-waves.[11,12] However, the preparation of clonidine used clinically is 0.38 mM out of the bottle, with a final concentration that is considerably lower (ie, 3.8 μM as estimated previously[1,6]). Consistent with these previous results, concentrations around those used clinically 0.3 to 30 μM produced no detectable inhibition of the A-wave ($EC_{50} = 1.3$ μM for $E_{max} = 0.01$) and a small (<50% of maximal) but significant ($P = 0.045$) inhibition of the C-wave. The $EC_{50}$ for this effect was 5.3 μM (Fig. 3). At non-neurotoxic concentrations approximating those used clinically, neither dexamethasone nor buprenorphine affected either A- or C-waves of the CAP (Fig. 2). As an additional control for the negative results obtained with dexamethasone and buprenorphine, we then tested epinephrine, an adjuvant that has been used more widely in clinical settings but which is thought to prolong the actions of local anesthetics via an extrinsic mechanism (ie, vasoconstriction) and therefore should not have a direct effect on the isolated

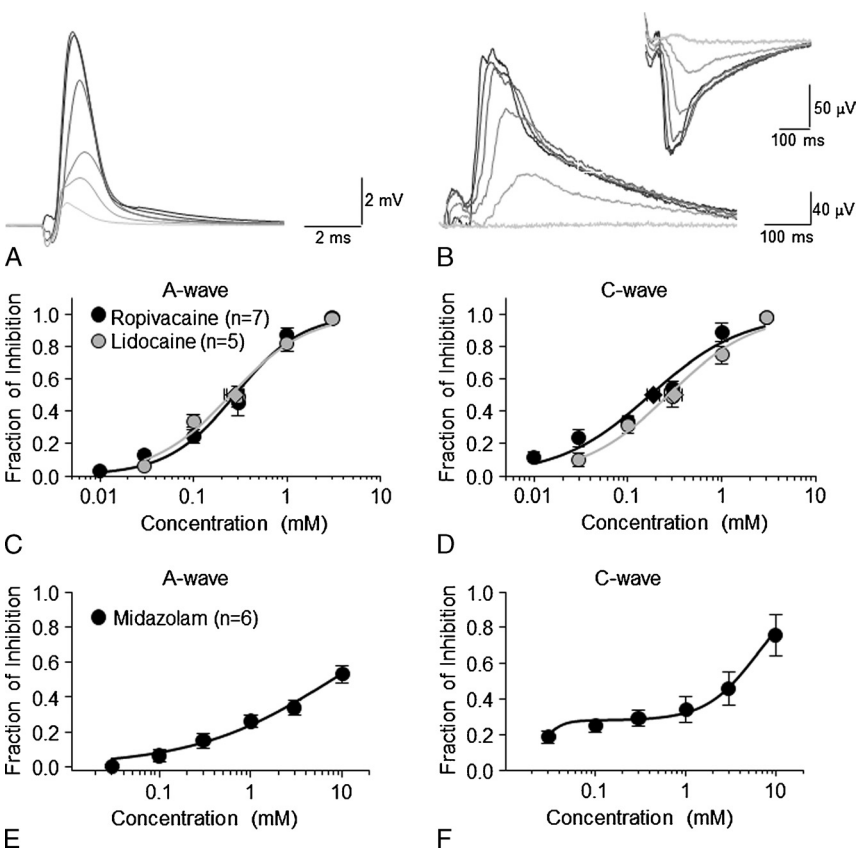


**FIGURE 1.** Traces from a representative recording session illustrating changes in rectified A-CAPs (A) and C-CAPs (B), associated with increased ropivacaine concentrations shown in C and D. Lighter shades indicate greater ropivacaine concentrations (0.01–3 mM). Insert in B shows C-waveforms before rectification. C, Concentration-response curves for changes in A-CAPs quantified as area under the curve (AUC) for a given trace as a function of ropivacaine (black circles) or lidocaine (gray circles) concentration. Data are presented as mean ± SEM for each concentration, whereas black and gray diamonds represent the concentrations that correspond to $EC_{50}$ values for ropivacaine (n = 7) and lidocaine (n = 5), respectively. D, Concentration-response curves for changes in C-CAP AUCs from the same nerves as in C. E and F, Concentration-response curves for midazolam A- and C-CAPs (n = 6).

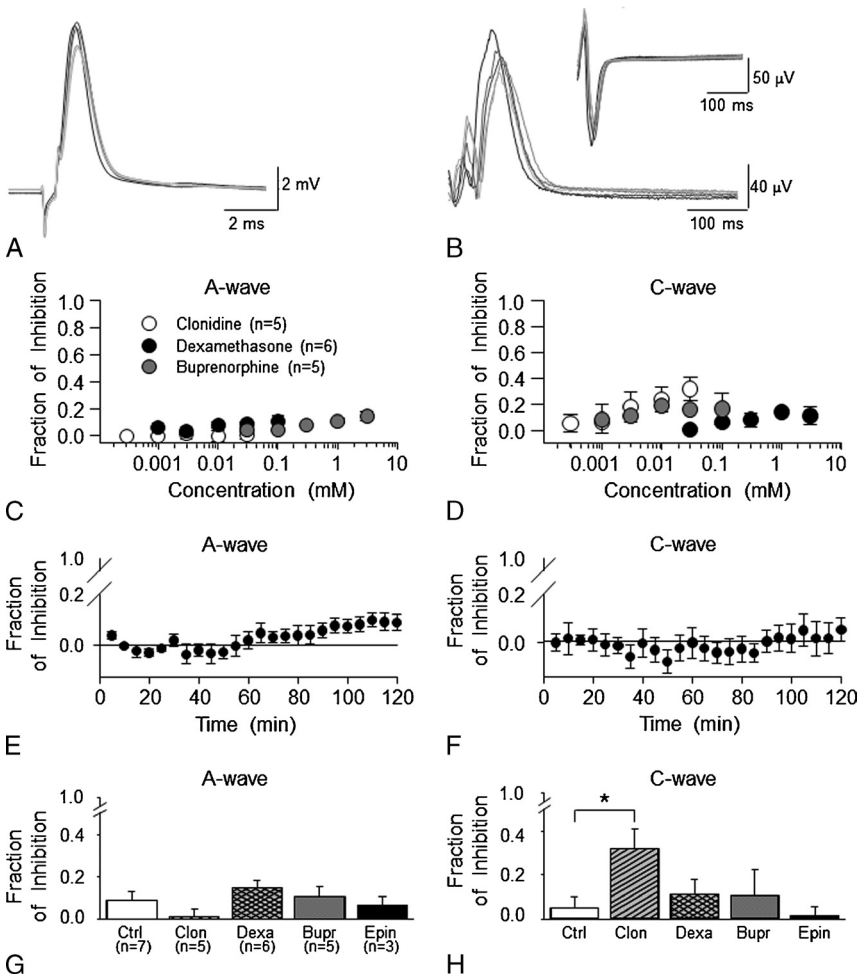


**FIGURE 2.** Traces from a representative recording session, illustrating changes in rectified A-CAPs (A) and C-CAPs (B), associated with increasing clonidine concentrations. Lighter shades indicate greater concentrations (0.3–30 µM) of clonidine. Insert in B shows C-waveforms before rectification. C, Concentration-response data for changes in A-CAP AUCs for clonidine (white), dexamethasone (black), and buprenorphine (gray). Data are presented as mean ± SEM for each concentration (clonidine n = 5, dexamethasone n = 6, buprenorphine n = 5). D, Concentration-response data for changes in C-CAP AUCs from the same nerves as in C. E and F, Baseline levels of A-AUC (E) and C-AUC (F) for 2 hours. G, Comparison of A-AUC maximal inhibition obtained from the last data points in C for each adjuvant and for epinephrine (n = 3) to the baseline data (n = 7), recorded for the same duration. H, Comparison of C-AUCs to baseline for the same nerves in G.

nerve. Consistent with our expectations, epinephrine had no detectable influence on A- and C-waves (Fig. 2). Taken together, our results suggest the mechanisms underlying the action of these adjuvant drugs are likely to be extrinsic to the peripheral axons.

## Effects of Coapplication of Clonidine-Buprenorphine-Dexamethasone on Local Anesthetic- or Midazolam-Induced Inhibition of A- and C-CAPs

With little evidence that clonidine, dexamethasone, or buprenorphine at clinically relevant concentrations were efficacious in blocking A or C components of the CAP, we next assessed the impact of the combination of clonidine + dexamethasone + buprenorphine on the actions of local anesthetics and of midazolam. We first examined the potency and efficacy of local anesthetic–induced block by comparing the effects of an $EC_{50}$ of local anesthetic alone or in combination with supraclinical concentrations of clonidine (30 µM), dexamethasone (1.3 mM), and buprenorphine (60 µM). The combination of these 3 adjuvant drugs alone had no detectable influence on the magnitude of either the A- or C-wave of the CAP and it did not significantly influence the block produced by $EC_{50}$ concentrations of ropivacaine (0.2 mM) or lidocaine (0.3 mM) (Fig. 3). Given the significant and potentially selective effect of midazolam on the C-wave, we next assessed the effect of a combination of clonidine + dexamethasone + buprenorphine on the block produced by midazolam (0.1 mM). As with the local anesthetics, these adjuvant drugs had no detectable influence on the block produced by midazolam (Fig. 3). Finally, as a control for the negative results obtained with clonidine + dexamethasone + buprenorphine, we assessed the impact of epinephrine on the potency of lidocaine-induced block of the CAP. Consistent with our expectations, epinephrine had no detectable influence on the potency of lidocaine (Fig. 3).

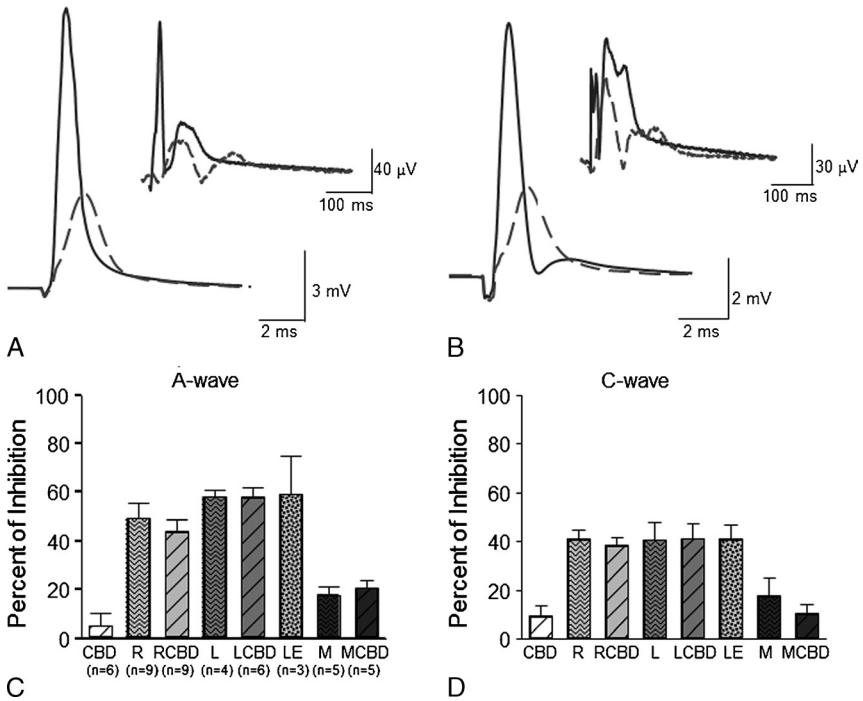


**FIGURE 3.** Addition of adjuvant drugs does not enhance local anesthetic– or midazolam-induced decreases in CAPs. A and B, Rectified A-CAP and C-CAP (inserts) traces before (solid lines) and after (dotted lines) 0.2 mM ropivacaine application in the absence (A) and presence (B) of clonidine-buprenorphine-dexamethasone (CBD) mixture. C and D, Drug-associated percentage decreases in A-AUCs (C) and C-AUCs (D). CBD, n = 6; R, ropivacaine alone, n = 9; RCBD, ropivacaine and CBD mixture, n = 9; L, lidocaine, n = 4; LCBD, lidocaine and CBD mixture, n = 6; LE, lidocaine and epinephrine, n = 3; M, midazolam, n = 5; MCBD, midazolam and CBD mixture, n = 5.

### Effect of the Coapplication of Clonidine-Buprenorphine-Dexamethasone on the Time Course of Recovery From Local Anesthetic–Induced Block of the CAP

Because perineural adjuvant drugs are primarily used with local anesthetics as a means to prolong the duration of local anesthetic–induced nerve block, we assessed the effect of the adjuvant drugs on the time course of recovery from local anesthetic block. Preliminary results with ropivacaine suggested that the time to 50% recovery at well over 12 hours was too long to have confidence in the viability of the preparation; therefore, we used the shorter-acting local anesthetic lidocaine for these experiments. An $EC_{50}$ of lidocaine was applied to the isolated nerve alone or in combination with the 3 adjuvant drugs (clonidine-buprenorphine-dexamethasone [CBD]). A stable block was produced for 10 minutes, after which all drugs were washed, and recovery of the CAP was monitored. The CBD adjuvant drugs had no detectable influence on the time courses of recovery of either A or C-waves of the CAP when applied with the $EC_{50}$ of lidocaine (Fig. 4). The time constants for recovery of the A-wave (41.91 ± 9.39 minutes for recovery from lidocaine alone and 41.12 ± 9.84 minutes for lidocaine + adjuvant drugs) and C-wave (32.85 ± 9.57 minutes for recovery from lidocaine alone and 39.84 ± 17.12 minutes for lidocaine + adjuvant drugs) did not differ significantly ($P > 0.05$ in both cases). To address the possibility that the lack of effect of adjuvant drugs on recovery from lidocaine is concentration dependent, we repeated the time course of recovery experiments with a concentration of 3 mM lidocaine that completely blocks both A- and C-waves. Consistent with the results obtained with an $EC_{50}$ of lidocaine, the CBD adjuvant combination had no significant ($P > 0.05$) influence on the time course of recovery from complete block of the sciatic nerve: the time constants of recovery were 24.41 ± 2.83 and 56.27 ± 21.08 minutes for the A-waves and 28.49 ± 3.61 and 48.83 ± 15.67 minutes for the C-waves for 3 mM lidocaine in the presence and absence of adjuvant drugs, respectively. Although not directly relevant to our central hypothesis, the CBD combination of adjuvant drugs had no detectable influence on the rate of onset of local anesthetic–induced block (not shown). Finally, as a control for the negative results obtained with CBD on the time course of recovery from lidocaine-induced block, we assessed the effect of epinephrine on the time course of recovery from lidocaine-induced block of the CAP. Consistent with our expectations, epinephrine had no detectable influence on the time course of recovery of lidocaine-induced block: the time constants of recovery were 31.99 ± 3.36 and 38.64 ± 9.72 minutes for A-waves and 28.13 ± 15.35 and 33.43 ± 8.21 minutes for C-waves for 0.3 mM lidocaine in the presence and absence of epinephrine, respectively.

## DISCUSSION

The purpose of this study was to determine (1) whether the commonly used adjuvant drugs clonidine, buprenorphine, dexamethasone, and midazolam have efficacy in the block of action potential propagation at clinically relevant concentrations by themselves and (2) whether these drugs directly influence local anesthetic–induced block of action potential propagation. The principal findings from this study are 3-fold. First, of the adjuvant drugs tested, only midazolam (and to a lesser extent clonidine) was efficacious in attenuating CAP propagation in the isolated sciatic nerve at clinically relevant concentrations. The potency and efficacy of the block of the C-wave for both

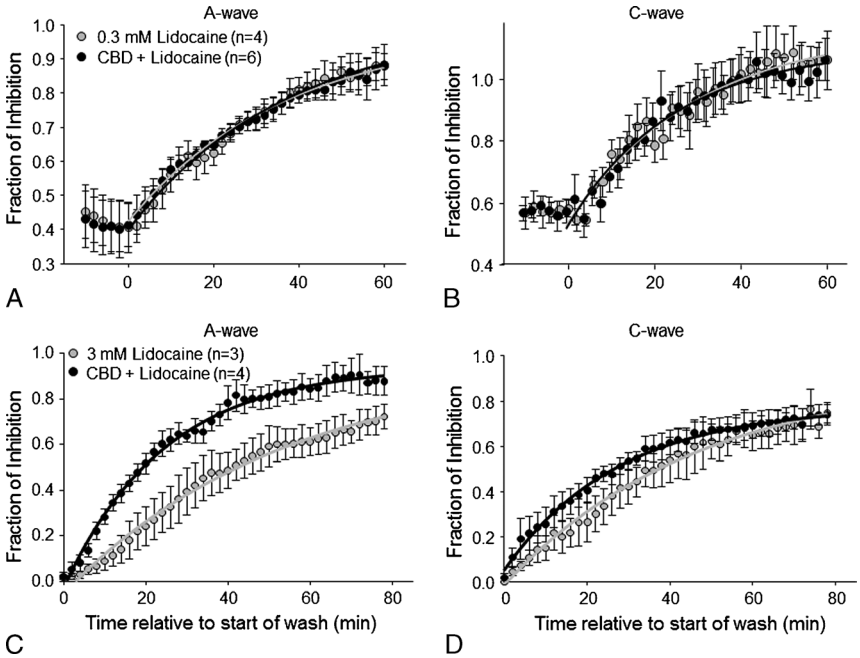


**FIGURE 4.** Presence of clonidine-buprenorphine-dexamethasone (CBD) does not influence washout kinetics of lidocaine (L). Changes in A-AUCs (A) and C-AUCs (B) plotted for 60 minutes after the start of the wash after 0.3 mM lidocaine (gray) or 0.3 mM lidocaine + CBD mixture (black) treatments. Changes in A-AUCs (C) and C-AUCs (D) plotted for 80 minutes after the start of the wash after 3 mM lidocaine (gray) or 3 mM lidocaine + CBD mixture (black) treatments. *P* = not significant for all comparisons.

midazolam and clonidine were significantly greater than those of the A-wave. Second, the combination of clonidine, dexamethasone, and buprenorphine, each at a concentration 10 times that used clinically, had no influence on the potency of local anesthetic–induced block of the CAP or the potency and efficacy of midazolam-induced block of the CAP. Third, and most relevant to the understanding of the clinical use of these adjuvant drugs, the combination of adjuvant drugs did not increase the time course of recovery from lidocaine-induced block of the CAP. This last observation raises the possibility that adjuvant drugs act indirectly to prolong the actions of local anesthetic–induced nerve block in vivo.

There are at least 3 aspects of the experimental design that may have impacted the results obtained and the conclusions drawn from them. First, all electrophysiologic experiments were conducted at room temperature. This was done because we sought to analyze the impact of anesthetics and adjuvant drugs on A- and C-waves in relative isolation, which is difficult to achieve at elevated (eg, body) temperatures, especially with relatively short nerves (<30 mm). If adjuvant drugs influence the actions of local anesthetics by affecting the interaction between anesthetics and voltage-gated $Na^+$ channels, and if this interaction were temperature dependent, it is possible that, by recording at room temperature, we failed to detect such an interaction. Second, these studies were conducted on sciatic nerves isolated from the rat, and data that support the use of adjuvant drugs to prolong the actions of local anesthesia had been collected in humans. Thus, species differences, in particular those specific to voltage-gated $Na^+$ channels, the primary target of local anesthetics, may have contributed to our failure to detect an influence of adjuvant drugs. Third, we were forced to perform our "recovery" experiments with lidocaine rather than ropivacaine because the time course for the recovery from ropivacaine alone (ie, >12 hours) precluded the ability to accurately measure an influence of adjuvant drugs. It is therefore possible that we have missed an influence of adjuvant drugs that are specific to the longer-acting ropivacaine.

Results of the present study suggest that the effects of midazolam are due to a direct action on the peripheral nerve. Although the actions of midazolam are not restricted to the gamma subunit containing A-type GABA ($GABA_A$) receptor,[13,14] we are not aware of evidence to suggest that this compound has any influence on voltage-gated $Na^+$ channel activity. Our own preliminary data on $Na^+$ currents in isolated sensory neurons (data not shown) suggest that, at the concentrations used in the present study, the blocking effects are not due to an action on $Na^+$ channels. Furthermore, although there is evidence that $GABA_A$ receptors are present[15] and functional[15,16] in peripheral nerve, the inability of midazolam to directly activate $GABA_A$ receptors argues against a $GABA_A$ receptor–mediated increase in chloride conductance as a mechanism for midazolam-induced block of the CAP. Of the known targets for midazolam, this leaves the peripheral benzodiazepine receptor as a potential mechanism of action, although additional data would be needed to both confirm this possibility and identify the link between the peripheral benzodiazepine receptor and CAP block.

One of the more interesting observations in the present study was the differential influence of midazolam on the A- and C-waves of the CAP. A selective C-fiber block would be ideal in the clinical setting, given that most nociceptive signals, particularly those associated with tissue injury, are carried by C-fibers.[17] Thus, such a selective block should result in the suppression of pain while leaving motor and proprioceptive fibers intact, thereby mitigating one of the major deleterious consequences of complete nerve block. Although these results are intriguing, our previous data[11] suggest that midazolam is toxic to primary afferents at concentrations used clinically.[1] Identifying the mechanism of midazolam-induced block, however,

may enable identification of other compounds that confer a selective C-fiber block in the absence of toxicity.

An isolated peripheral nerve in vitro preparation was used in the present study because we sought to determine whether the interaction between local anesthetics and adjuvant drugs involved mechanisms intrinsic to the peripheral nerve. The failure to detect an influence in isolated nerves suggests that the nature of the interaction involves mechanisms extrinsic to the peripheral nerve. For example, clonidine may be acting at $\alpha_2$-adrenergic receptors on sympathetic postganglionic terminals, or local vasculature, or via an "off-target" mechanism, such as the hyperpolarization-activated cation current ($I_h$)[18] in a cell type that is subsequently able to influence local anesthetic actions on the primary afferent. Similarly, dexamethasone may reduce inflammation associated with anesthetic administration, thereby indirectly prolonging anesthetic effects. Given the presence of opioid receptors in both local[19] and recruited immune[20] cells, buprenorphine could be acting indirectly via a comparable mechanism. Future in vivo studies will be needed to explore these possibilities.

In summary, these results indicate that adjuvant drugs reported to prolong the actions of local anesthetic–induced block of peripheral nerve do so by indirect mechanisms. Identification of these mechanisms may enable the development of novel approaches to both prolong the actions of local anesthetics and increase the therapeutic window of these compounds.

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## REFERENCES

1. Williams BA, Murinson BB, Grable BR, Orebaugh SL. Future considerations for pharmacologic adjuvants in single-injection peripheral nerve blocks for patients with diabetes mellitus. *Reg Anesth Pain Med*. 2009;34:445–457.
2. Costantini R, Affaitati G, Fabrizio A, Giamberardino MA. Controlling pain in the post-operative setting. *Int J Clin Pharmacol Ther*. 2011;49:116–127.
3. Neal JM. Effects of epinephrine in local anesthetics on the central and peripheral nervous systems: neurotoxicity and neural blood flow. *Reg Anesth Pain Med*. 2003;28:124–134.
4. Whiting DJ, Williams BA, Orebaugh SL, Toshok RR. Case report: postoperative analgesia and preserved motor function with clonidine and buprenorphine via a sciatic perineural catheter. *J Clin Anesth*. 2009;21:297–299.
5. Vieira PA, Pulai I, Tsao GC, Manikantan P, Keller B, Connelly NR. Dexamethasone with bupivacaine increases duration of analgesia in ultrasound-guided interscalene brachial plexus blockade. *Eur J Anaesthesiol*. 2010;27:285–288.
6. Williams BA, Hough KA, Tsui BY, Ibinson JW, Gold MS, Gebhart GF. Neurotoxicity of adjuvants used in perineural anesthesia and analgesia in comparison with ropivacaine. *Reg Anesth Pain Med*. 2011;36:225–230.
7. Cummings KC 3rd, Napierkowski DE, Parra-Sanchez I, et al. Effect of dexamethasone on the duration of interscalene nerve blocks with ropivacaine or bupivacaine. *Br J Anaesth*. 2011;107:446–453.
8. Ilfeld BM, Morey TE, Enneking FK. Continuous infraclavicular perineural infusion with clonidine and ropivacaine compared with ropivacaine alone: a randomized, double-blinded, controlled study. *Anesth Analg*. 2003;97:706–712.
9. Ilfeld BM, Morey TE, Thannikary LJ, Wright TW, Enneking FK. Clonidine added to a continuous interscalene ropivacaine perineural infusion to improve postoperative analgesia: a randomized, double-blind, controlled study. *Anesth Analg*. 2005;100:1172–1178.
10. Gold MS, Reichling DB, Hampl KF, Drasner K, Levine JD. Lidocaine toxicity in primary afferent neurons from the rat. *J Pharmacol Exp Ther*. 1998;285:413–421.
11. Butterworth JF, Strichartz GR. The $\alpha_2$-adrenergic agonists clonidine and guanfacine produce tonic and phasic block of conduction in rat sciatic nerve fibers. *Anesth Analg*. 1993;76:295–301.
12. Leem JW, Choi Y, Han SM, Yoon MJ, Sim JY, Leem SW. Conduction block by clonidine is not mediated by $\alpha_2$-adrenergic receptors in rat sciatic nerve fibers. *Reg Anesth Pain Med*. 2000;25:620–625.
13. Beurdeley-Thomas A, Miccoli L, Oudard S, Dutrillaux B, Poupon MF. The peripheral benzodiazepine receptors: a review. *J Neurooncol*. 2000;46:45–56.
14. DalBo S, Nardi GM, Ferrara P, Ribeiro-do-Valle RM, Farges RC. Antinociceptive effects of peripheral benzodiazepine receptors. *Pharmacology*. 2004;70:188–194.
15. Carlton SM, Zhou S, Coggeshall RE. Peripheral GABA(A) receptors: evidence for peripheral primary afferent depolarization. *Neuroscience*. 1999;93:713–722.
16. Reis G, Pacheco D, Francischi J, Castro M, Perez A, Duarte I. Involvement of $GABA_A$ receptor–associated chloride channels in the peripheral antinociceptive effect induced by $GABA_A$ receptor agonist muscimol. *Eur J Pharmacol*. 2007;564:112–115.
17. Meyer RA, Ringkamp M, Campbell JN, Raja SN. Peripheral mechanisms of cutaneous nociception. In: McMahon SB, Koltzenburg MK, eds. *Wall and Melzack's Textbook of Pain*. Philadelphia, PA: Elsevier; 2006:3–34.
18. Kroin JS, Buvanendran A, Beck DR, Topic JE, Watts DE, Tuman KJ. Clonidine prolongation of lidocaine analgesia after sciatic nerve block in rats is mediated via the hyperpolarization-activated cation current, not by α-adrenoreceptors. *Anesthesiology*. 2004;101:488–494.
19. Hermens JM, Ebertz JM, Hanifin JM, Hirshman CA. Comparison of histamine release in human skin mast cells induced by morphine, fentanyl, and oxymorphone. *Anesthesiology*. 1985;62:124–129.
20. Busch-Dienstfertig M, Stein C. Opioid receptors and opioid peptide-producing leukocytes in inflammatory pain—basic and therapeutic aspects. *Brain Behav Immun*. 2010;24:683–694.

 